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Research article

Muscle specialization in the squid motor system

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Summary

Although muscle specialization has been studied extensively in vertebrates, less is known about the mechanisms that have evolved in invertebrate muscle that modulate muscle performance. Recent research on the musculature of squid suggests that the mechanisms of muscle specialization in cephalopods may differ from those documented in vertebrates. Muscle diversity in the development and the evolution of cephalopods appears to be characterized by modulation of the dimensions of the myofilaments, in contrast to the relatively fixed myofilament dimensions of vertebrate muscle. In addition, the arrangement of the myofilaments may also be altered, as has been observed in the extensor muscle fibres of the prey capture tentacles of squid and cuttlefish, which show cross-striation and thus differ from the obliquely striated pattern of most cephalopod locomotor muscle fibres. Although biochemical specializations that reflect differences in aerobic capacity have been documented previously for specific layers of the mantle muscle of squid, comparison of protein profiles of myofilament preparations from the fast cross-striated tentacle fibres and slow obliquely striated fibres from the arms has revealed remarkably few differences in myofilament lattice proteins. In particular, previous studies using a variety of SDS-PAGE techniques and peptide mapping of the myosin heavy chain were unable to resolve differences in the myosin light and heavy chains. Since these techniques cannot exclude the presence of a highly conserved variant that differs in only a few amino acids, in this study semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of myosin heavy chain messenger RNAs (mRNAs) from the cross-striated tentacle and obliquely striated arm muscle fibres was conducted. This analysis showed that a previously reported alternatively spliced isoform of the squid myosin motor domain is present only in low abundance in both muscle types and therefore differential expression of the two myosins cannot explain the difference in contractile properties. It thus appears that modulation of the contractile properties of the musculature of squid and other cephalopods occurs primarily through variation in the arrangement and dimensions of the myofilaments.

Key words: squid, muscle, myosin, RT-PCR, myofilament.

Introduction

Specialization of muscle fibres is an important and widespread component of the evolution of motor systems. The performance of muscle fibres, such as their maximum velocity, maximum tension, twitch duration and endurance, is frequently modulated to suit their particular role in locomotion, movement and support. The ways by which muscle fibre performance is altered have been investigated in detail in vertebrates (e.g. Moore and Schachat, 1985; Rome and Klimov, 2000; Rome et al., 1988; Rome and Lindstedt, 1998; Schiaffino and Reggiani, 1996; Sweeney et al., 1988; Schachat et al., 1987; Schachat et al., 1990; Wigmore and Dunglison, 1998) and arthropods (Cochrane et al., 1972; Costello and Govind, 1983; Gronenberg et al., 1997; Günzel et al., 1993; Jahromi and Atwood, 1969; Marden, 2000; Marden et al., 1998; Marden et al., 1999; Marden et al., 2001; Stephens et al., 1984; Stokes et al., 1975; Syme and Josephson, 2002). Less is known, however, about the mechanisms of muscle specialization in invertebrates in general. The goal of this paper is to review, briefly, recent investigations into the mechanisms of muscle specialization in squid and other cephalopods and to describe experiments designed to explore the potential role of alternative splicing of the squid myosin heavy chain in the modulation of muscle performance.

Squid prey capture

The unusually rapid strike of the tentacles of squid during prey capture represents an excellent model system for examining the mechanisms of muscle specialization for high shortening velocity. During the prey strike, the eight arms flare open and the two tentacles are rapidly elongated so that the terminal portion of the tentacles, which is equipped with suckers, contacts and attaches to the prey (Chen et al., 1996; Fields, 1965; Hurley, 1976; Kier, 1982; Kier and van Leeuwen, 1997; LaRoe, 1971; Lee et al., 1994; Neill and Cullen, 1974; Nicol and O'Dor, 1985). Kinematic analysis of high-speed cine films of the strike shows that the elongation occurs in only 20–40 ms, the peak strain in the tentacle stalks ranges from 0.43 to 0.8, the peak longitudinal strain rates range from 23 to 45 s^{-1}, the peak velocity is greater than 2 m s^{-1} and the peak acceleration is approximately 250 m s^{-2} (Kier and van Leeuwen, 1997). The tentacle strike is thus a remarkably rapid movement.

Arm and tentacle muscle

The musculature responsible for this remarkable performance in elongating the tentacles is the transverse muscle mass of the tentacles. This muscle mass is serially homologous with the transverse muscle mass of the arms, which is responsible for bending and supporting the arms (Kier, 1982), and the tentacle

transverse muscle mass probably evolved from musculature similar to that found in the arms (Kier, 1996; Kier and Thompson, 2003). The current understanding of the evolution of coleoid cephalopods suggests that the ancestral coleoid cephalopod possessed ten arm-like appendages. In the line that gave rise to the octopuses, one pair of arms was lost, and in the line that gave rise to the decapod cephalopods (squid and cuttlefish) one pair of arms became modified as tentacles (von Boletzky, 1993; Naef, 1921–1923). Thus, by comparing the transverse muscle fibres in the tentacles with the transverse muscle fibres in the arms, we can gain insight into the ways in which the tentacle musculature has been specialized for high shortening velocity and fast contraction.

Although the arrangement of the transverse muscle mass in the arms and in the tentacles is similar, previous studies of the ultrastructure have revealed striking differences (Kier, 1985; Kier, 1991; Kier and Curtin, 2002). The ultrastructure of the transverse muscle fibres of the arms is similar to that of most of the other musculature in cephalopods; the fibres of the transverse muscle mass are obliquely striated with relatively long thick filaments [7.4 μm in *Loligo pealei* (Kier and Curtin, 2002)]. Although the myofilaments of obliquely striated muscle are oriented parallel to the long axis of the fibre as they are in other striated muscles, they are not lined up in register across the fibre, and instead are staggered, forming an oblique or helical alignment. The muscle fibres of the transverse muscle mass of the tentacles, by comparison, are highly unusual for cephalopods. These fibres show cross-striations and have thick filaments that are unusually short [0.7 μm in *Loligo pealei* (Kier and Curtin, 2002); Fig. 1].

Functional implications of muscle structure

The ultrastructural differences between the fibres of the transverse muscle masses of the arms and the tentacles have important implications for the shortening velocity and peak tension generated by these two fibre types. Because the myofilaments and sarcomeres of the transverse muscle mass of the tentacles are much shorter than those of the transverse muscle mass of the arms, the tentacle fibres include more elements in series, per unit length of muscle fibre, compared with the arm fibres. Since the shortening velocity of elements in series is additive, the ultrastructure of the tentacle fibres suggests that they should have a much higher unloaded shortening velocity than the arm fibres (Huxley and Simmons, 1972; Josephson, 1975; van Leeuwen, 1991). This prediction was tested in a study that compared the contractile properties of isolated fibre bundle preparations from the transverse muscle mass of the arms and the transverse muscle mass of the tentacles (Kier and Curtin, 2002). Since the thick filaments of the tentacle fibres were found to be approximately one-tenth the length of the thick filaments of the arm fibres, the maximum shortening velocity (V_{max}) of the tentacle fibres was predicted to be ten times as high. The estimates of V_{max} of the tentacle fibres were indeed found to be ten times as high as those of the arm fibres, based on fitting Hill's equation to the force–velocity relationship for isotonic contraction of the two fibre types (tentacle: $15.4\pm1.0\ L_0\ s^{-1}$; arm: $1.5\pm0.2\ L_0\ s^{-1}$; means ± s.d., where L_0 is the length at which peak isometric force was recorded). While the shorter thick filaments result in a higher shortening velocity for the tentacle fibres, one can predict that they will have a lower peak tension because fibres with shorter thick filaments have fewer cross-bridges operating in parallel, per half sarcomere, resulting in lower tension (Josephson, 1975). The peak tension measured in the transverse muscle fibres of the tentacle was found to be much lower than that of the arm (Kier and Curtin, 2002).

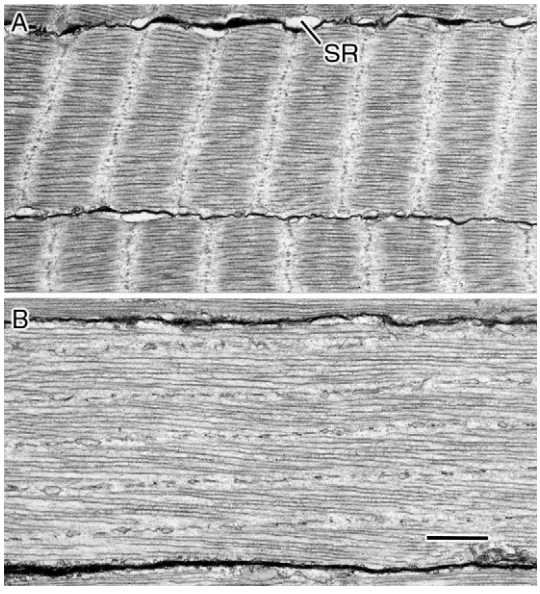

Fig. 1. Transmission electron micrographs of longitudinal sections of muscle fibres from the transverse muscle mass of the tentacle (A) and arm (B) of *Loligo pealei*. Note the short sarcomeres and short thick filaments of the tentacle fibres compared with the longer thick filaments of the obliquely striated arm fibres. SR, sarcoplasmic reticulum. Scale bar, 1 μm. From Kier and Curtin (Kier and Curtin, 2002).

The implications of the ultrastructural specializations of the tentacle fibres for the overall performance of the tentacles during the strike were also explored with a theoretical model (van Leeuwen and Kier, 1997). In this analysis, a forward dynamics model of the tentacular stalk was developed that incorporated the correct orientation, arrangement and amount of muscle. The model used as inputs the dimensions of the tentacle, the passive and active muscle properties, myofilament lengths and activation of the muscle fibres. The predictions of the model were in agreement with the previously described kinematic observations from high-speed cine films of the prey strike. The model allowed exploration of the effects of changes in the myofilament dimensions on the peak velocity and peak kinetic energy of the tentacle strike. In particular, the thick filament length giving peak tentacle extension velocity predicted by the model was remarkably close to the actual measurements of thick filament length taken from electron micrographs (van Leeuwen and Kier, 1997; Kier and Curtin, 2002).

Muscle biochemistry

The myofilament protein composition of fibres from the transverse muscle masses of the arm and tentacle was also compared in order to ascertain the potential role of biochemical specialization in the differences in contractile properties of the two muscle fibre types (Kier and Schachat, 1992). Myofilament preparations from the arm and tentacle fibres were compared using SDS-PAGE. Although identical techniques have been used to document the extensive biochemical heterogeneity of vertebrate muscle fibre types, these techniques showed little evidence of differences in contractile protein isoforms between the arm and tentacle fibres. The relative amount of the protein paramyosin, however, was greater in the arm fibres. In addition to investigations of myofilament preparations using SDS-PAGE, myosin heavy chains from the arm and tentacle fibres were purified and compared using cyanogen bromide and V8 protease peptide mapping techniques; these analyses also failed to

resolve any differences between muscle fibres from the arm and tentacle transverse muscle masses (Kier and Schachat, 1992).

Although the biochemical comparison of the arm and tentacle fibres employed identical techniques to document the extensive molecular heterogeneity of vertebrate muscle fibre types, the techniques are unlikely to resolve highly conserved protein isoforms that differ in only a few amino acids. Indeed, a study that sequenced the myosin heavy chain from the funnel retractor muscle of *Loligo pealei* detected two isoforms that are alternatively spliced transcripts from the squid muscle myosin heavy chain gene (Matulef et al., 1998). The two alternatively spliced myosin mRNAs for isoforms A and B differ in the ATP-binding loop in the myosin head. In contrast to the sequence of myosin A, the alternatively spliced exon incorporated into the myosin B mRNA introduces several amino acid substitutions and shortens the variable region of the ATP-binding loop by five amino acids (Matulef et al., 1998). Given the location on the myosin head, there is the possibility that the contractile properties could be affected. The present study was designed to determine whether the two isoforms are differentially expressed in the fibres of the transverse muscle masses of the arm and tentacle and thus might play a role in determining the difference in contractile properties.

Materials and methods

To determine the relative abundance of mRNA for the two myosin heavy chain isoforms, semi-quantitative RT-PCR was performed using primers that spanned the alternatively spliced region. The procedure was analogous to that used to determine the relative abundance of alternatively spliced troponin T mRNA populations in mammalian (Briggs and Schachat, 1993; Briggs and Schachat, 1996) and avian skeletal muscle (Schachat et al., 1995). The RT-PCR primers were designed so that myosin A would generate a 204 nucleotide (nt) product, and myosin B would generate a 189 nt product (15 nt shorter due to the deletion of five amino acids in its ATP-binding loop).

Specimens of *Loligo pealei* (Lesueur 1821) were supplied by the Marine Biological Laboratory, Woods Hole, MA, USA. The specimens were flash frozen in liquid nitrogen and entire arms and tentacles were removed and kept frozen on dry ice during the dissections. Samples of the transverse muscle mass from the arms and from the tentacles were removed and transferred immediately to liquid nitrogen. The samples were then pulverized in a mortar and pestle chilled with liquid nitrogen. RNA was isolated from each muscle sample using the Qiagen RNeasy kit (Valencia, CA, USA) and their protocol for fibrous tissue. RT-PCR was performed using an iCycler™ as described by Briggs and Schachat (Briggs and Schachat, 1993; Briggs and Schachat, 1996). The forward primer was 5′-AGCTTGGCTGGAAAGAAAGATAA-3′; the reverse primer was 5′-CAGCACCGGCAATTTTACCTT-3′. Following 22 cycles of amplification, the products were resolved by polyacrylamide gel electrophoresis in Tris-borate-EDTA buffer on a BioRad precast 10% polyacrylamide Ready Gel™ (Hercules, CA, USA). The products were visualized by staining with SYBER Green and the image captured on a UVP BioChemi™ (UVP, Inc., Upland, CA, USA) imaging system. Quantitative analysis was performed using the public domain NIH Image program (developed at the US National Institutes of Health by Wayne Rasband and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Results

Polyacrylamide gel electrophoresis of the RT-PCR products revealed low levels of myosin B mRNA in both muscle fibre types (Fig. 2). Quantitative densitometric analysis revealed that myosin A mRNA is overwhelmingly abundant in fibres from both the arm and the tentacle transverse muscle masses; myosin B mRNA makes up less than 5% of the total myosin heavy chain mRNA in the arm and less than 10% in the tentacle.

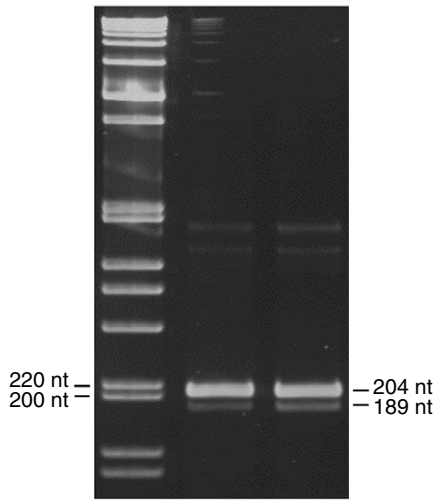

Fig. 2. Photograph of polyacrylamide gel (10% acrylamide) showing the relative abundance of myosin A (204 nucleotides, nt) and myosin B (189 nt) mRNAs following RT-PCR of RNA from muscle fibres of the transverse muscle mass of the arm (middle lane) and the tentacle (right lane). The 1 kb ladder (left lane) confirms the size of the products.

Discussion

The low levels of the alternatively spliced myosin heavy chain variant and the lack of a significant difference in the levels of the variant between the arm and the tentacle muscle fibres are consistent with our previous studies that suggest that ultrastructural differences account for the difference in contractile properties of the arm and tentacle muscle fibres (Kier, 1985; Kier, 1991; Kier, 1996; Kier and Curtin, 2002; Kier and Schachat, 1992; van Leeuwen and Kier, 1997). It is likely that the relative abundance of the two mRNAs reflects the level of the myosins present in fibres because the difference between the two alternatively spliced mRNAs lies in an internal sequence (Matulef et al., 1998) distant from the 3′ or 5′ untranslated sequences that are implicated in translational control (Lewin, 2007), and previous research on mammalian muscle has revealed a direct relationship between mRNA levels and contractile protein expression (Kropp et al., 1987; Streher et al., 1986). Moreover, it is unclear where or whether the myosin B isoform is a predominant species as this is the first report on the relative abundance of either its mRNA or protein. Consequently, these results provide additional evidence that high shortening velocity was achieved by a reduction in the dimensions of the myofilaments and sarcomeres of the fibres of the transverse muscle mass of the tentacles, rather than by changes in the biochemistry of proteins of the myofilament lattice. This mechanism of specialization provides an interesting contrast to previous work on muscle specialization in vertebrates, where the dimensions and the arrangement of the myofilaments are relatively invariant (Eisenberg, 1983; Hoyle, 1983; Offer, 1987) but a range of isoforms of the various myofilament proteins is expressed. It is this biochemical heterogeneity that is responsible for much of the variation in the contractile properties of vertebrate muscle fibre types.

A change in myofilament dimensions has thus played the primary role in the specialization for fast contraction of the tentacle fibres. It is unclear, however, whether this is a general mechanism of muscle specialization that is exploited by squid and other cephalopods. Although a range of thick filament lengths have been reported in the previous studies of the ultrastructure of cephalopod muscle, we have relatively little comparative information on the contractile properties and biochemistry of the fibres (Amsellem and Nicaise, 1980; Cloney and Florey, 1968; Dykens and Mangum, 1979; Florey, 1969; Gonzalez-Santander and Socastro Garcia-Blanco, 1972; Hochachka et al., 1978; Jensen and Tjønneland, 1977; Kawaguti, 1963; Kawaguti and Ikemoto, 1957; Kling and Schipp, 1987; Milligan et al., 1997; Nicaise and Amsellem, 1983; Schipp and Shäfer, 1969; Socastro, 1969; Ward and Wainwright, 1972). A recent study by Thompson and Kier (Thompson and Kier, 2006) on the development of the mantle musculature in squid (*Sepioteuthis lessoniana*) provides some evidence that changes in filament length correlate with changes in velocity during ontogeny. Video recordings of escape jets of squid during ontogeny showed that the peak velocity of mantle contraction was highest in newly hatched squid and declined during ontogeny [hatchling: 8.6±2.1 $L\ s^{-1}$; juvenile 1: 4.8±1.2 $L\ s^{-1}$; juvenile 2: 3.8±1.7 $L\ s^{-1}$; young 2: 3.8±0.55 $L\ s^{-1}$; means ± s.d. (Thompson and Kier, 2006)]. The thick filament length, measured on electron micrographs of the superficial mitochondria-rich (SMR, analogous to vertebrate red fibres) mantle fibres averaged 1.0 μm in newly hatched squid and 1.9 μm in juveniles. The central mitochondria-poor (CMP, analogous to vertebrate white muscle fibres) mantle fibres averaged 1.0 μm in hatchlings and 1.5 μm in juveniles. Thus, the shortest thick filaments appear to be present in the mantle muscle fibres that show the highest shortening velocity, although it should be emphasized that the mantle contraction rates were measured in swimming animals. The observed ontogenetic change in thick filament length is also interesting in the context of scale effects on jet locomotion. If we make the reasonable assumption of approximately isometric growth in squid, then the hatchlings will be relatively stronger since muscle force scales with length squared, but mass scales with length cubed. Increasing shortening velocity by decreasing thick filament length is thus a good option for the hatchlings because scaling may compensate for the concomitant reduction in muscle stress.

Additional studies are needed to document the contractile properties of cephalopod muscle in controlled conditions using conventional muscle mechanics techniques. Although aspects of the biochemistry and ultrastructure of squid mantle and fin muscle fibres relevant to their aerobic capacity have been studied previously (Bone et al., 1981; Kier, 1989; Mommsen et al., 1981), the biochemistry of the myofilaments of the mantle and fin muscle fibres, and indeed other cephalopod muscle fibres, has not yet been analysed. A comparative analysis is needed of myofilament biochemistry from a diverse sample of fibres that differ in contractile properties.

Cross-striated fibres with short thick filaments have also been observed in the transverse muscle mass of the tentacles of the cuttlefish *Sepia officinalis* (W.M.K., unpublished observations). *Sepia* also rapidly elongates its tentacles to capture prey in a manner quite similar to that of squid described above. The ultrastructure of the fibres of the transverse muscle mass is similar to that of squid and these fibres also possess short thick filaments (0.9 μm). Additional analysis of the biochemistry of these fibres would be of great interest as well.

Change in striation pattern

The implications of the change in myofilament length for shortening velocity are clear, but it is less obvious why a change in striation pattern evolved in the fast-contracting tentacle fibres (Bone et al., 1995). In principle, a decrease in thick filament length and hence the 'sarcomere' length should increase the shortening velocity of a muscle fibre, whether it be obliquely striated or cross-striated. Why, then, did the cross-striated pattern evolve in the transverse musculature of the tentacles of squid and cuttlefish? This question cannot be answered definitively at this point, but there are several interesting implications of the striation pattern for the function of the muscle fibre that may have played a role in its evolution.

In soft-bodied invertebrates, the range of elongation and contraction of the musculature is typically not limited in the manner seen in most animals with joints and rigid skeletal elements, and the deformations may exceed the normal range of elongation and contraction of cross-striated fibres. For example, the range of contraction of the mantle musculature of hatchling and juvenile squid (*Sepioteuthis lessoniana*) during the escape jet was measured to be 40–60% from hyperinflation to full contraction (Thompson and Kier, 2001). This is a greater range of length change than is typical for cross-striated fibres, which typically do not exceed 30–40% (Burkholder and Lieber, 2001). The mechanisms that allow obliquely striated muscle fibres to produce force over large length changes are still not entirely clear, although there is some evidence that the staggered arrangement may allow the thick and thin filaments to 'change partners' when pulled beyond overlap during extension of the fibre (Lanzavecchia, 1977; Lanzavecchia, 1981; Lanzavecchia and Arcidiacono, 1981; Miller, 1975).

Analysis of the biomechanics of the tentacles of squid and cuttlefish has shown that the range of elongation and contraction that occurs in the fibres of the transverse muscle mass during the tentacle strike is smaller – approximately 30% shortening from rest length, a range that could be accommodated by cross-striated fibres (Kier, 1982; van Leeuwen and Kier, 1997). Thus, if the oblique striation pattern is not required in the fibres of the transverse muscle mass of the tentacles, what are the potential selective pressures that might favour the evolution of cross-striation? One possibility may relate to the implications of the oblique stagger of thick and thin filaments for cross-bridge interaction with the thin filaments. Because of the displacement of the thick and thin filaments relative to one another in the oblique arrangement, a greater number of cross-bridges will interact with a thin filament on one side of a thin filament *versus* the other (Fig. 3). This non-symmetrical interaction of cross-bridges with thin filaments is a potential problem for obliquely striated fibres with either long or short thick filaments

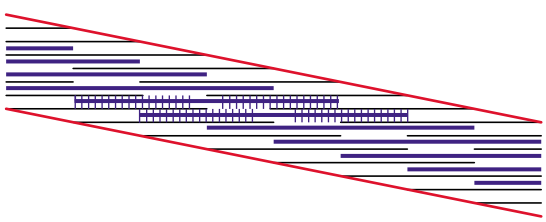

Fig. 3. Schematic diagram of a portion of an obliquely striated muscle fibre showing the unsymmetrical interaction of the cross-bridges with thin filaments due to the stagger of myofilaments. The thin filaments are black, the thick filaments (including two with myosin cross-bridges) are blue and the Z-elements are red.

but, at a given angle of striation, the non-interacting cross-bridges represent a larger proportion of the total in fibres with short thick filaments compared with those with long thick filaments. This inefficiency in cross-bridge interaction is eliminated in a cross-striated fibre and this may therefore have been a selective advantage in the evolution of cross-striation of the tentacle transverse muscle fibres. It is worth emphasizing that the longitudinal muscle fibres that shorten the tentacles following a strike are subjected to elongations of up to 100% and show the typical cephalopod oblique striation pattern (Kier, 1985; Kier, 1991).

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